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Search for relationships among the hemolytic, phospholipolytic, and neurotoxic activities of snake venoms

(crotoxin components/bungarotoxins/phospholipase A2)

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ABSTRACT **Several snake venom neurotoxins are larger and more complex than the well-studied group of postsynaptic toxins exemplified by α-bungarotoxin. Several of these, exemplified by β-bungarotoxin, show phospholipase A2 activity (phosphatide 2-acylhydrolase, EC 3.1.1.4) when tested in the presence of detergents. The high hemolytic activity of crotoxin, the neurotoxin of *Crotalus durissus terrificus*, in the presence of lecithin has been attributed to this activity. The phospholipase A2 activity of several snake venom proteins has now been compared under the physiological conditions of the hemolysis tests.**

It appears that only the basic component of crotoxin, B, is enzymatically active, and that its activity is not inhibited by component A under these conditions, or in the presence of deoxycholate. Phosphatidylserine is found to be digested more readily than egg white phosphatidylcholine; and also causes hemolysis in conjunction with much lower levels of crotoxin. In neither case is calcium required or stimulating.

Phospholipase from *Crotalus adamanteus*, which is not neurotoxic, digests phosphatidylcholine more rapidly than does crotoxin, but phosphatidylserine more slowly; yet it is slightly less active than crotoxin in the hemolysis test with phosphatidylcholine, and much less with phosphatidylserine. The digestion of several phospholipids by either enzyme fails to release the expected protons in the absence of detergents at 37°.

β-Bungarotoxin, highly neurotoxic, has negligible phospholipase A2 activity in the absence of detergents, and is almost nonhemolytic in conjunction with all phospholipids tested.

Binding studies with ^{125}I-labeled compounds show that rabbit erythrocytes and ghosts have much greater affinity for crotoxin than for β-bungarotoxin and do not bind *Crotalus adamanteus* phospholipase. The crotoxin complex is split in the course of binding, with only component B, the hemolytic component, becoming bound. It appears that the role of component A may be to diminish the nonspecific binding tendency of component B.

Our data appear to be consistent with the concepts that affinity to membranes, particularly to specific sites on synaptic membranes, is the critical requirement for β type neurotoxicity, and that this property, at least in some instances, has evolved from phospholipase A2 enzymes, but does not necessarily require retention and expression of enzymatic activity.

When the neurotoxin of the Brazilian rattlesnake (*Crotalus durissus terrificus*) was isolated in pure crystalline form in 1938 and was found to also carry the indirect (i.e., lecithin-dependent) hemolytic activity of that venom (1), it was then suggested (and has since been reiterated) that the ability of crotoxin to attack phospholipids might represent the basis of its neurotoxic potency.

When, many years later, after crotoxin had been shown to consist of two proteins (2), the surprising finding was made that high neurotoxicity required the presence of both components (3–5), while in regard to hemolytic activity the larger and basic component, crotoxin B, alone was fully active, this seemed to deny any direct relationship between the neurotoxicity and the hemolytic as well as, presumably, the phospholipolytic activity of this venom.

Snake venom neurotoxins had at that time come to be classified as belonging to either of two groups differing in their site of action. Most of these toxins, exemplified by α-bungarotoxin, acted postsynaptically by blocking the acetylcholine receptor sites; a less numerous group, exemplified by β-bungarotoxin, seemed to act presynaptically. The exact mode by which the "β type" toxins block neuromuscular impulse transmission remains uncertain. Because several of these neurotoxins show phospholipase A2 activity (phosphatide 2-acylhydrolase, EC 3.1.1.4), the hypothesis that this enzymatic activity plays a major role in their neurotoxicity was again considered (6, 7). That this cannot be a simple cause and effect relationship is evident because several phospholipases have been isolated from the same and other snake venoms and from other sources that are not at all neurotoxic. Also, the most toxic β type snake venom toxin, taipoxin (8), was reported to have very low phospholipase A2 activity (9)‡, and that of β-bungarotoxin is detectable only in the presence of detergents (6, 10).

As far as crotoxin is concerned, it has shown properties intermediate between α and β type neurotoxins (12, 13). It has been reported that the crotoxin complex, as well as its basic component (which by itself is of low toxicity), shows phospholipase A2 activity in the presence of detergents or at high temperature (14, 15), which is in line with our finding of the indirect hemolytic activity of these, but not the acidic component (3, 4). The present study deals with attempts to further test the questions to what extent crotoxin, β-bungarotoxin, and *Crotalus adamanteus* phospholipase are enzymatically active under biological conditions and to what extent this action can be correlated with these toxins' indirect hemolytic and/or neurotoxic potency. Further, binding studies are reported which indicate that crotoxin B is bound to a much greater extent to erythrocyte membranes than are the other proteins; and that crotoxin A may diminish this nonspecific binding tendency of B.§

MATERIALS AND METHODS

Crotoxin, crotoxin A, and crotoxin B were prepared according to Hendon and Fraenkel-Conrat (3), usually by using both

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‡ Recently, high phospholipase A2 activity was reported for taipoxin when acting in the presence of deoxycholate on L-α-phosphatidylcholine purified on an aluminum oxide column (10). Taipoxin has not yet been tested for hemolytic and phospholipase activity under the conditions used in this paper.

§ We prefer the terms A and B for the components of crotoxin, rather than crotapotin and *Crotalus durissus terrificus* phospholipase used by Habermann *et al.* and Breithaupt (14, 15), because there also exists in the same venom an acidic nontoxic phospholipase.

cationic and anionic cellulose chromatography, both in 6 M urea. The α- and β bungarotoxins were gifts from E. Bennett (Laboratory of Chemical Biodynamics, University of California, Berkeley). All other chemicals and enzymes were commercial preparations.

Hemolytic Activity. Hemolytic activity was assayed with washed rabbit erythrocyte suspensions in 0.9% NaCl adjusted so that 100% hemolysis gave an A_{540} reading of about 1.0 for the final solution. The phospholipid substrates, after evaporation of any organic solvent with a stream of nitrogen, were suspended by stirring with 0.5 ml of 0.9% saline, and emulsified with the aid of a vortex mixer. The typical reaction mixture in 1 ml of 0.9% NaCl contained: 0.2–10 μg of "enzyme," 0.1 mg of lipid substrate, the erythrocyte suspension, and 1 μl of 0.5 or 1 M calcium chloride. The mixture was incubated at 37° for 30 min, followed by 10–20 min at 0–4°; after centrifuging at low speed in a bench-top centrifuge for 5 min, the absorbance of the supernatant was read for released hemoglobin at 540 nm. The cooling played a major role in completing lysis near threshold levels of lysolecithin. That this was a physical phenomenon, not a continuing enzymatic action, was shown by centrifuging incompletely lysed samples at 25–28°, and washing them with saline. Only upon cooling of their suspension did these cells lyse. Data on the effect of temperature shift-down in the action of lysolecithin on liposomal membranes were reported by Inoue and Kitagawa (16).

Assay for Phospholipase A2. Assays for phospholipase A2 activity were performed in principle according to de Haas *et al.* (17) and Halpert *et al.* (18), using a well-shaken suspension of an egg yolk in 100 ml of H_2O as substrate (0.7 ml), 2.5–8 mM sodium deoxycholate, and 1–30 mM $CaCl_2$ (usually 2.5 and 20 mM, respectively), in a total volume of 2.1 ml, maintained at pH 8.1 ± 0.05 by means of a pH-stat at about 25°. Because uptake of alkali (0.02 M) upon addition of the enzyme (0.1–25 μg) diminished with time, the consumption during the first 5-min period was used as a measure of enzymatic activity. Because this was not strictly proportional to the amount of enzyme solution used, conclusions are based on relative amounts of enzyme needed to obtain a digestion rate of about 2 μmol/5 min. To simulate the conditions of hemolysis, digestions were also performed at 37° for 30 min without deoxycholate. Lipids were extracted according to Martin *et al.* (19). Aliquots of the lipid residue, redissolved in methanol/chloroform (1:1) were subjected to thin-layer chromatography on Chromagram silica sheets (Eastman Kodak Co., Rochester, NY) according to Hughes and Frais (20). Other aliquots were dried, emulsified with saline, and tested for lysophospholipids by hemolysis.

Binding Tests. Binding assays were done with ^{125}I-labeled proteins, prepared in principle according to Hunter and Greenwood (21), and having 100–200 × 10^5 cpm/μg. Rabbit erythrocytes were prepared according to Hanahan and Ekholm (22); it was found that 8–10 washes were needed to clear the membranes of hemoglobin, and we have used Ringer solution for the last two washes.

The binding capability of these preparations for ^{125}I-labeled crotoxin B, the same complexed with crotoxin A, and labeled β-bungarotoxin and *C. adamanteus* phospholipase were tested, using about 0.4 mg of ghost protein or 7–10 mg of erythrocyte protein, as estimated by the method of Lowry *et al.* (23), and 0.1–30 μg of toxin. The reaction mixture in 1 ml of Ringer solution was held at room temperature (21°) for 60 min. Erythrocyte membranes were then centrifuged at 13,000 rpm in a Sorvall with a 34SS head for 20 min. The pellet was washed three times by resuspending it in 2 ml of Ringer solution and recentrifuging at 13,000 rpm. Erythrocytes were centrifuged at low speed for 5 min. After washing, the pellets were resuspended, decanted into new tubes, and assayed for radioactivity. The decanting separated the actual cell- or membrane-bound from the tube- (glass or plastic) bound radioactivity, the latter being a major amount when dealing with crotoxin B. Binding is expressed as percent of the added radioactivity and as picomoles of toxin bound/mg of protein. An alternate method is a modification of the method of Oberg and Kelly (24) using Bio-Gel A-5 [0.8 × 24 cm, equilibrated with modified Ringer solution (2.5 mM Ca^{2+}) + 0.05% NaN_3, flow rate 5 ml/hr, 1-ml fractions] with the protein eluting in tubes 5 and 6 and the bulk of the radioactivity after tube 11.

Table 1. Phospholipase A2 activity of various phospholipases*

Enzyme	Phosphatidyl-choline	Egg yolk
Crotoxin complex	0.68	0.70
Crotoxin B	0.85	0.70
Crotoxin A followed by B†	0.77	0.80
β-Bungarotoxin	0.56	0.61
C. adamanteus phospholipase	14.4	4.6

* Alkali consumption (in presence of 30 mM Ca^{2+} and 8 mM deoxycholate), in μmol/μg of enzyme during the first 5-min period using 14 mg (20 μmol) of phosphatidylcholine or about 0.5% of an egg yolk. Most of the data are averages of duplicate tests done the same day. At other times higher and lower rates were observed, with β-bungarotoxin giving as much as 6 times the digestion rate given by crotoxin B and complex (which were always similar) and *C. adamanteus* phospholipase as much as 29 times.

† Crotoxin A (13 μg) caused no alkali consumption; when B was added (4 μg) the indicated consumption was observed.

RESULTS

Phospholipase A2 Activity. When phospholipase activity was determined in the pH-stat at pH 8.2 in the usual way in the presence of sodium deoxycholate and calcium (e.g., 2.5 and 20 mM), crotoxin complex and crotoxin B were found similarly active, β-bungarotoxin slightly less active, and purified *C. adamanteus* phospholipase an order of magnitude more active (Table 1). α-Bungarotoxin and crotoxin A were inactive. Crotoxin A did not inhibit the activity of B. This result regarding crotoxin components agrees with data recently reported by Breithaupt (15) who, however, found such inhibition when activities were tested in the absence of deoxycholate at 55°. Similar relative activities were observed with whole egg yolk and egg white phosphatidylcholine. However, phosphatidylserine was digested much more readily in the absence of added Ca^{2+}, both in the presence and absence of deoxycholate. The inhibitory effect of Ca^{2+} on the digestion of phosphatidylserine, due to insolubility of its calcium salt, has recently been discussed by Dennis and collaborators (25).

Phospholipids were also digested under the conditions favorable for hemolysis assays, that is, without deoxycholate for 30 min at 37°. These experiments were done either under conditions similar to those used for hemolysis tests or at 10- to 20-fold higher substrate concentration with either the same or correspondingly increased Ca^{2+} concentration. The results, illustrated by a few examples on Table 2, were not greatly different. It appeared that little if any alkali was taken up at pH 8.2 with the above substrates, and if the pH used was that of the erythrocyte suspension (7.2), it slowly drifted up to pH 7.6 during the 30-min test. Nevertheless, lysophospholipids could be produced in significant amounts under these conditions, usually mounting to 10–20% digestion. This was shown by extraction of the lipids, which were then tested for direct hemolytic activity, and fractionated by thin-layer chromatography. These tests relied on comparing the digestion mixture with

Table 2. Phospholipase A2 assays under physiological conditions*

Enzyme, μg	Substrate, μmol	Additives, μmol	Lysophospholipid,† μmol
Crotoxin, 10	PC, 5.5‡	Ca, 40	0.8
Crotoxin, 10	PC, 5.5	Ca, 4	0.8
C. adamanteus PLA, 10	PC, 5.5‡	Ca, 40	1.6
β-Bungarotoxin, 20	PC, 5.5‡	Ca, 40	0.01
Crotoxin, 1 (pH 7.4)§	PC, 0.55	Ca, 4	0.08
C. adamanteus PLA, 1 (pH 7.4)§	PC, 0.55	Ca, 4	0.08
Crotoxin, 10	PS, 4.6	None	1.0
C. adamanteus PLA, 5	PS, 2.7	None	0.3
β-Bungarotoxin, 20	PS, 2.7	None	0.1

* At 37° ± 1°, 30 min; pH 8.2 unless otherwise indicated. Reaction mixtures are 2 ml. PC and PS signify egg white phosphatidylcholine and phosphatidylserine, and PLA signifies phospholipase.

† These values are approximations, derived from comparisons of the amount of the lipid extracts of the digest needed to cause 50% hemolysis under standard conditions (see Table 3) compared to pure L-α-lysophosphatidylcholine; of the latter, 8–16 μg are needed with different samples of erythrocytes. These values were in many instances confirmed within ±30% by lysophospholipid analyses by thin-layer chromatography (see *Materials and Methods*). Titration in the pH-stat usually gave insignificant uptake of 0.02 M NaOH (<0.3 μmol) during the digestion period; only with crotoxin acting on phosphatidylserine was there definite alkali uptake (~0.5 μmol).

‡ Synthetic dipalmitoyl lecithin gave under the same conditions for crotoxin, *C. adamanteus* phospholipase, and β-bungarotoxin, respectively: titration values of 1, 2.6, and 0.2 μmol; and about 1.3, 5, and 0.2 μmol of lysolecithin by hemolysis tests.

§ Typical conditions of hemolysis test at twice normal substrate and Ca concentration.

Table 3. Hemolysis assays

Enzyme	Approximate amount needed for 50% lysis, μg/ml: With PC* (+Ca)†	With PS (−Ca)†
Crotoxin	0.25	0.05
Crotoxin B	0.15–0.3‡	0.025–0.05†
Crotoxin A	>10	>20
C. durissus terrificus phospholipase§	0.5	0.1
C. adamanteus phospholipase	0.5	2.5
α-Bungarotoxin	>50	
β-Bungarotoxin	50	75

* Fifty percent hemolysis is obtained with dipalmitoyl lecithin using 1 μg of *C. adamanteus* enzyme, but 20 μg of crotoxin are needed.

† Ca^{2+} (0.5 or 1 mM) is required for efficient hemolysis of phosphatidylcholine (PC); with phosphatidylserine (PS) and phosphatidylethanolamine, hemolytic activity is similar, or slightly higher in the absence of added Ca^{2+}. Phosphatidylethanolamine is less sensitive to crotoxin than is phosphatidylserine, and is even slightly less sensitive than phosphatidylcholine, requiring crotoxin at about 0.5 μg/ml for 50% lysis.

‡ Assays of crotoxin B show more variability than others, possibly because of its great tendency to adhere to surfaces, particularly at low concentrations.

§ This preparation from Sigma appears to be unfractionated crotoxin or *C. durissus terrificus* venom, because it shows all the properties, including high toxicity, of the crotoxin complex.

known amounts of commercial L-α-lysophosphatidylcholine in regard to their capability to hemolyze (for which about 15 μg of lysolecithin was needed for half lysis of 1 ml of fresh erythrocytes suspension) or to give, on thin-layer plates, small spots with I_2 vapor, the size and color of which indicated the amount of material in semiquantitative manner. These methods generally gave approximately similar results, indicating that sufficient amounts of lysophospholipids appear to be formed under the conditions of the hemolysis tests to account for the hemolytic action of crotoxin and *C. adamanteus* phospholipase. It must thus be concluded that, in the absence of detergents, enzymatically liberated fatty acids can remain partly undissociated at pH 8.2, be it due to hydrogen bonding to the glycerol OH group or micelle-structural constraints, and that pH-stat titrations thus do not necessarily represent a full measure of phospholipase activity under such conditions. In contrast to these observations, synthetic dipalmitoyl lecithin, though in suspension, gave normal titration values upon digestion with *C. adamanteus* phospholipase, even in the absence of deoxycholate, and it was actually digested more readily by all the enzymes tested, yet showed the same order of activities observed with deoxycholate (*C. adamanteus* phospholipase ≫ crotoxin ≫ β-bungarotoxin) (Table 2).

Hemolytic Activity. The hemolytic activity of crotoxin on rabbit cells requires the presence of a phospholipid. With phosphatidylcholine, Ca^{2+} is also required, although at much lower concentration than customarily used for phospholipase assay (0.5 versus 20 mM). Synthetic dipalmitoyl lecithin, though digestible in the pH-stat, proved much less active in the hemolysis test than the other phospholipids tested, when crotoxin was used, but more active with *C. adamanteus* phospholipase. This paradox was not investigated further.

With phosphatidylserine and phosphatidylethanolamine the presence of Ca^{2+} is not necessary, and in certain instances it is unfavorable. Phosphatidylserine, if used in the usual excess (0.1 mg/ml) in the absence of Ca^{2+}, actually causes hemolysis at one-fifth the crotoxin concentration required for phosphatidylcholine. When the toxin is used in excess the two phospholipids are similarly active, about 20 μg being needed, without and with Ca^{2+}, for phosphatidylserine and phosphatidylcholine, respectively. This corresponds to the amount of pure lysolecithin needed (about 15 μg) to achieve the same results without an enzyme.

Under standard assay conditions with excess phospholipid, crotoxin A is inactive, while crotoxin B and the complex are similarly highly active (Table 3). Purified *C. adamanteus* phospholipase is about half as active as crotoxin, and β-bungarotoxin causes hemolysis only at a 200-fold level of phospholipid. Because β-bungarotoxin was also found to show no significant phospholipase activity under these conditions in the absence of deoxycholate, its lack of hemolytic activity supports the lysolecithin mechanisms of indirect hemolysis. However, the fact that purified *C. adamanteus* phospholipase, while more active as a phospholipase, is less active as a hemolytic agent than crotoxin, suggests that lysolecithin formation may not represent the only effect of crotoxin in causing hemolysis. It appears probable that crotoxin also has a direct effect on the erythrocyte membrane, which may render it more sensitive to lyso-compounds and particularly to lysophosphatidylserine. For this reason binding studies were undertaken with the ^{125}I-labeled enzyme and/or hemolytic snake venom components.

Binding of Snake Venom Components to Erythrocytes or Ghosts. When the bindings by erythrocytes of ^{125}I-labeled crotoxin B, A, and the complex, labeled β-bungarotoxin, and labeled *C. adamanteus* phospholipase were compared, it became evident that considerably more of the added crotoxin B than β-bungarotoxin was bound, and that the non-neurotoxic

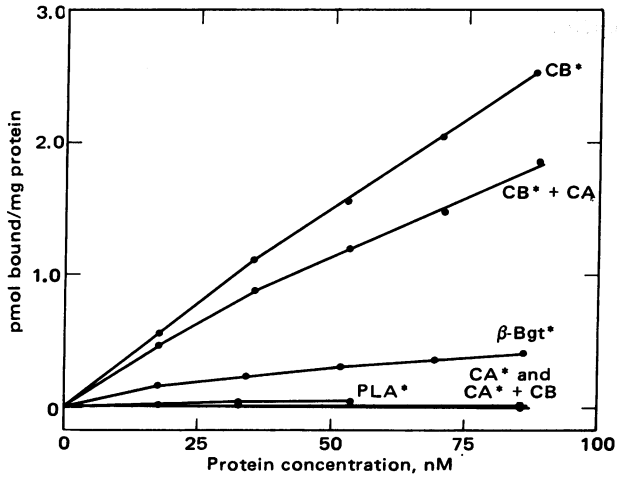

FIG. 1. Binding of venom components by erythrocytes. Binding to erythrocytes of ^{125}I-labeled proteins, identified by *; CA and CB represent crotoxin components A and B, β-Bgt represents β-bungarotoxin, and PLA represents *C. adamanteus* phospholipase. All were iodinated at the level of 1 ± 0.5 mol/mol and all retained 60–100% of their original biological activity or potential.

venom phospholipase and crotoxin A were not appreciably bound (Fig. 1). Similar data were obtained in presence of high Ca^{2+} (2.5 mM) and without Ca^{2+}. The amounts of binding increased in all instances in approximate proportion to the amount added, with little indication of reaching a saturation level. Thus, these data represent a measure of the various affinities, or possibly uptake capabilities, rather than a measure of specific binding sites. The data can be most simply expressed in terms of percentages of the added radioactivity that became bound; with increasing amounts of the labeled materials these ranged, in a typical experiment, from 22 to 18% for crotoxin B, from 13 to 8% for the complex, from 2.9 to 2.0% for β-bungarotoxin, and from 0.8 to 0.4% for *C. admanteus* phospholipase. No significant amounts of crotoxin A were bound whether it was used alone or in complex with unlabeled B. Thus, it appears that the crotoxin complex is dissociated upon contact with the B-binding membrane. The lesser binding of the complex as compared to B may be due to the complex having to become dissociated in the course of binding of the B component.

The high binding of labeled crotoxin was not chased by subsequent treatment of the erythrocytes with a 100-fold excess of the unlabeled compound, while pretreatment with the latter greatly diminished but did not abolish the binding of the labeled compounds. Also under these conditions, the net binding of labeled crotoxin B failed to reach a saturation level, while the much lower binding of β-bungarotoxin appeared to do so at a level of 0.17 pmol/mg.

That the relatively low binding of β-bungarotoxin was not due to selection of noniodinated molecules was demonstrated by a serial dilution experiment with unlabeled β-bungarotoxin, which showed that the unlabeled compound was not bound preferentially. The fact that all these iodo derivatives retained their original toxicity and/or phospholipase activity also supports the conclusion that β-bungarotoxin and particularly *C. adamanteus* phospholipase are bound less to the erythrocytes than crotoxin B is.

Binding to erythrocyte ghosts has given rather similar data in all but one respect, namely, that the radioactivity of the crotoxin B in complex form is bound to ghosts to a similarly high extent as is free crotoxin B (40–50%), while with erythrocytes twice as much B is bound or taken up when free as compared to the complex. The difference between the crotoxin samples and β-bungarotoxin and *C. adamanteus* phospholipase is at least as marked with ghosts as with erythrocytes, with only 6% and 1% of these proteins being bound, respectively, to the ghosts. Saturation levels are again not reached over a 16-fold range in toxin addition, and little of the radioactivity is chased by 100- to 1000-fold excess unlabeled crotoxin B, while pretreatment leads to somewhat less binding of the labeled material (6%), neither procedure showing clear evidence of saturation of binding sites. In terms of pmol/mg of protein, ghosts bind about 30-fold more than intact erythrocytes. These results confirm that we are dealing with surface binding, and not uptake by the erythrocytes.

To ascertain the validity of our technique of determining the binding, a few key experiments were repeated, using a gel sieve method similar to that used by Oberg and Kelly (24), although with agarose rather than Sephadex G-200. Under these conditions, with β-bungarotoxin, 64% and 90%, respectively, of the ghost protein and the radioactivity was recovered, and of the radioactivity 2.6% was bound to the protein, which corresponded to 3.7 pmol/mg, compared to about 7 pmol/mg found by the centrifugation method. The corresponding data for crotoxin B were 47 and 61% recovery, and 22% of the label bound to the ghosts, which corresponded to 43 pmol/mg, as compared to about 52 pmol/mg found by the centrifugation method. Thus, the absolute binding levels are slightly lower and the difference between crotoxin and β-bungarotoxin are somewhat greater with the column method. These binding studies clearly show that crotoxin B has a much greater affinity for erythrocytes and their membranes than do the other phospholipases used in these studies.

The finding that component A was released as B became bound to the erythrocyte membrane made it appear possible that the biological role of component A might be to counteract the nonspecific binding tendency of B, and thus favor its ability to reach its target, the neuromuscular junction. To test this hypothesis, we injected the same amount (2.5 μg, 361×10^5 cpm) of ^{125}I-labeled crotoxin B, either alone or complexed with unlabeled crotoxin A, into the tail veins of two groups of three mice. After 3 hr, when the mice receiving complex were dead, the others were killed and the diaphragms and abdominal muscle of both groups were washed, dried, and analyzed for radioactivity. It appeared that upon injection of crotoxin B alone 20 and 23×10^{-4}% were found in diaphragm and abdominal muscle, respectively, while upon injection of the complex 46 and 53×10^{-4}% were found per mg of dried muscle.

DISCUSSION

There is now much interest in the phospholipase A2 activity of several presynaptically acting neurotoxins, and its role in this toxic action (e.g., refs. 6 and 7). Crotoxin's mode of action has not yet been clearly identified, but it certainly shows some features of presynaptic action (26, 27), and the fact that it has phospholipase A2 activity supports its classification with the β type neurotoxins. However, the relationship between phospholipase A2 activity and neurotoxicity is certainly not simple and direct, because the most active enzyme preparations from snake venoms (28) and other sources are not neurotoxins, and the most active snake neurotoxin, taipoxin (8), was under standard conditions (17, 18) reported to have very low phospholipase A2 activity (9).‡ Our present comparative study of the phospholipase and indirect hemolytic activity of crotoxin, β-bungarotoxin, and *C. adamanteus* phospholipase further illustrates the complexity of the system.

Crotoxin and its B component, the one highly toxic and the other barely so (3, 22, 29), carry similar indirect hemolytic activity and show similar phospholipase A2 activity when tested in the presence of deoxycholate or at elevated temperature.

However, under physiological conditions, these proteins show in the pH-stat little if any splitting of the same egg white lecithin that serves well as a source of lysolecithin in the hemolysis test. This paradox was explained when we found that digestion of phospholipids under gentle conditions can occur without release of titratable protons. Thus, crotoxin's action on egg white lecithin and phosphatidylserine is in part masked, but analyses for lyso-products indicates that these are probably formed in sufficient amounts to account for the hemolytic potential of crotoxin.

C. adamanteus phospholipase also is an indirect hemolytic agent, and it also can digest lecithin without releasing a proton under physiological conditions. Its enzymatic activity, particularly with deoxycholate, is higher than that of crotoxin, and yet it is less active in indirect hemolysis tests. An explanation for this is suggested by the results of the binding studies discussed below.

β-Bungarotoxin differs from the two proteins discussed above in that it has almost no hemolytic activity, and this is accounted for by its actual failure to digest phospholipids at a significant rate under physiological conditions. Thus, it appears quite dubious to attribute any neurobiological function to β-bungarotoxin's phospholipase activity, demonstrable only in solutions containing unphysiologically high Ca^{2+} and detergent.

Focusing now on the substrate, we find that phosphatidylserine is digested much more readily than are phosphatidylcholine and phosphatidylethanolamine if crotoxin (complex and B) is used, but not with the other two proteins; the digestion of phosphatidylserine and phosphatidylethanolamine requires no calcium. In line with the preference of crotoxin for phosphatidylserine, hemolysis is achieved by much less crotoxin in its presence than is required with the other phospholipids. The functional significance of this specificity is obscure.

Focusing finally on the lipid-rich membranes that probably represent the actual target of the neurotoxic phospholipases, we have begun by studying as a model system the binding affinity of erythrocytes and their ghosts for the same three snake venom components, all ^{125}I labeled. These studies have yielded both a new paradox and an interesting new finding. The paradox is that, while crotoxin is bound to erythrocyte membranes much more than the nonhemolytic β-bungarotoxin, the hemolytic *C. adamanteus* enzyme is not bound significantly. A possible interpretation is that the latter is not a membrane-targeted, but an exoenzyme, while β-bungarotoxin is a membrane-targeted enzymatically inactive neurotoxin, and crotoxin is both enzymatically and surface active. This is further supported and illustrated by the finding that of crotoxin only the B component becomes bound, A being released in the process. Preliminary results obtained by one of us (T.-W.J.) in collaboration with J.-P. Changeux and C. Bon indicate that the same is true also for the binding of crotoxin components by *Torpedo* electroplax.

It has been shown that the interaction of crotoxin A and B leads to considerable conformational change (30). It is also well known that the tendency of crotoxin B to bind to all surfaces is greatly diminished by complex formation. One is thus tempted to hypothesize that the role of crotoxin A is to diminish the nonspecific binding of the actual toxin, B, so that enough of it can reach the nerve end plates to paralyze the snake's victim. This hypothesis appears to be supported also by the experiments in which we injected into the tail vein of mice either ^{125}I-labeled crotoxin or the same amount of labeled B component alone, and determined the amount of radioactivity in their diaphragm and abdominal muscle. The finding of over twice as much label in the muscles when the complex was administered supports for A the role of a "chaperone," as was recently suggested also for the β and γ components of taipoxin (8).

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